Causality behind biological robustness - a game theoretic approach to quantify negative entropy.

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Abstract

Biological systems have negative entropy in them. Here we propose an objective scheme to quantify the precise amount of negative entropy, present in an extremely important (and arguably the most well-studied) biochemical pathway; namely, the TCA cycle. Our approach is based on the computational implementation of two-person non-cooperative finite zero-sum game between positive entropy and negative entropy. Evolution of TCA cycle concentrations (for the first 100 minutes) provided the template of description of pure strategy for negative entropy in a biological system; whereas a matrix of random number of same dimension could establish the framework to describe pure strategy of positive entropy representing the physico-chemical universe. Biochemical analogue of Nash equilibrium condition between these two players, could unambiguously provide a quantitative marker that describes the 'edge of life' for TCA cycle. Difference between concentration-profiles prevalent at the 'edge of life' and biologically observed TCA cycle, could quantitatively express the precise amount of negative entropy present in a typical biochemical network. We show here that it is not the existence of mere order, but the synchronization profile between ordered fluctuations, which accounts for biological robustness. An exhaustive sensitivity analysis could identify the concentrations, for which slightest perturbation can account for enormous increase in positive entropy. Since our algorithm is general, the same analysis can as well be performed on larger networks and (ideally) for an entire cell, if numerical data for concentration is available. These results assume paramount importance not only because its numerical description of the enigmatic negative entropy; but also, due to the enormous (possible) benefit that it can offer to systemic view of drug discovery and the nascent field of synthetic biology.

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Introduction :

We start our argument with an observation from a series of immortal statements.

Some 135 years ago, in 1875, Boltzmann reasoned - "The general struggle for existence of animate beings is not a struggle for raw materials. These, for organisms, are air, water and soil, all abundantly available; nor for energy which exists in plenty in any body in the form of heat, but a struggle for entropy, which becomes available through the transition of energy from the hot sun to the cold earth."[1] Almost 70 years down the line, Schrodinger, in 1944, felt for an appropriate scheme of physico-chemical origin to model the spatial and temporal processes occurring within the boundaries of a cell. He constructed the question categorically in his inimitable style - "how can the events in space and time which take place within the spatial boundary of a living organism be accounted for by physics and chemistry?" He continued, "the obvious inability of present-day physics and chemistry to account for such events is no reason at all for doubting that they can be accounted for by those scientists"[2]. Some two decades after Schrodinger visionary talk, NASA had chosen James Lovelock (amongst a group of scientists) to make a theoretical life detection system that can search out for life on Mars. To Lovelock, the basic question was "What is life, and how should it be recognized?"; for which his answer was - "I'd look for an entropy reduction, since this must be a general characteristic of life"[3]. Finally, a popular Biochemistry textbook, in the dying years of the last century, asserted - "living organisms preserve their internal order by taking from their surroundings free energy, in the form of nutrients or sunlight, and returning to their surroundings an equal amount of energy as heat and entropy".[4]

We, in 2009, propose an algorithm here to quantify that enigmatic 'entropy reduction' in numerical terms; by describing the emergent synchronization profile(SP) from the biochemical pathways and comparing it with its possible magnitude in the (non-biological) physico-chemical universe. This comparison was carried out by resorting to two person non-cooperative finite zero-sum game. Although sporadic attempts have been made to apply game theoretic constructs to understand problems of biochemistry[5,6], these were largely confined to the paradigms of describing optimization procedures in pathway levels. Here, on the contrary, without attempting to optimize the yield from any part of the pathway, we were interested to measure the amount of negative entropy in any biological system. To be more precise, our goal was to relate (and biological robustness with the amount of negative entropy (and if possible, hypothesize a 'negative entropic' origin of biological robustness). To achieve this, we wanted to measure 'negative entropy'.

It assumes paramount significance at this point to clarify what precisely is implied by our use of the words 'negative entropy'. Within every living biological cell, thousands of macromolecules interact between themselves to construct a perfect harmony that is displayed by the degree of superb synchronization prevalent amongst the couplings between these pathways. What makes this synchronized orchestration so remarkable is the fact that almost all (if not all) the units (concentration of macromolecules) that create such symphony, operate within individual ranges or specified-intervals of their own; instead of maintaining a fixed magnitude over the time-scale. This implies, that the nature of mapping between these units suffers from an inherent uncertainty. Yet,

at the end of the day, more often than not these uncertainties are routinely overcome and from the top-down perspective, a perfect deterministic harmony emerges from, what would seem to be, an essentially uncertain mapping between several concentrations, from a bottom-up perspective. A quantification of the probability of emergence of the aforementioned "perfect deterministic harmony", is what will be referred to as 'negative entropy' throughout the length of this paper.

To objectively understand the nature and causality behind robustness in maintenance of this superb synchronization between individual biochemical pathways, we require a model to measure the "negative entropy" that ensures such orchestration, without ignoring the uncertain mapping. However, instead of attempting to "take the bull by its horn", we attempted to quantify the same from the opposite approach. That is, we measured the 'negative entropy' by quantifying the amount of 'positive entropy', required to ensure that the system discards its biological nature and behaves like a physico-chemical system. Owing to availability of experimental data, we conducted the study on perhaps the most most well-studied of all the biochemical systems, namely the TCA cycle. Although a TCA cycle, merely by itself, cannot produce the "perfect deterministic harmony" talked earlier, it is arguably the most conserved biochemical unit along the course of evolution; which implies that it must surely be endowed with seeds of possibilities that may produce the "perfect deterministic harmony". Hence, the choice of our model system, viz. the TCA cycle, can (roughly) be called a miniaturized version of a complete biological system with respect to profiling of negative entropy. Measurement of 'negative entropy' by quantifying the amount of 'positive entropy' to make a TCA cycle non-biological, is achieved by subjecting the TCA cycle to a steady source of proportionate perturbation. By measuring the amount of deviation of any state of the system from the thermodynamic equilibrium, we could categorically measure the negative entropy content of the biological system in that particular state.

Biological systems are embedded within the physico-chemical space-time. However, any particular biological system and the physico-chemical surrounding of it, never shares an one-to-one mapping (this is true even for the trees; because the temperature gradient, hygrometric parameters etc, continuously change around them). The efficiency with which any biological system adjusts itself to the varying physico-chemical space-time around it is described by biological adaptation. Phenomenon of adaptation describes the capability of biological systems to re-organize their filtering mechanism, so as to stave off the physico-chemical (positive) entropy from entering and mixing with the biological (negative) entropy. This filtering scheme translates to game theoretic parlance as 'optimal strategies'[7,8]. But here, instead of describing the evolution of such strategies, our motivation was to measure, :
First : The difference in positive and negative entropies at any snapshot of the process and describe it with a computationally implementable framework.

Second : to follow the locus of negative entropy (contained in the pathway) in its route towards the 'edge of life', which characterizes the zero-sum equilibrium point after a game between positive and negative entropy was played.

Game theoretic studies of biochemical systems is not new. Variegated approaches under game-based philosophies have been applied to numerous systems with varying efficiencies[8-14]. The present work differs from the existing spectrum because, here

without resorting to evolutionary game theoretic constructs, we are applying standard procedure of non-cooperative finite zero-sum game principle to ascertain the amount of negative entropy a particular pathway (or a set of them) is embodying. The equilibrium point of a finite zero-sum antagonistic game between positive entropy (entropy of physico-chemical universe) and negative entropy (entropy of biological system, embedded within the physico-chemical universe) will indicate us the very 'edge of life'. This 'edge of life' can be described by the synchronization profile between (time-dependent and context-dependent) macromolecular concentrations in a dying person. Whereas, for the entropic profile of a comparable system representing physico-chemical universe, the 'edge of life' (EOL) will be given by the point at which rigor-mortis is setting in. Since, we are relating EOL to the equilibrium reached after the positive entropy versus negative entropy game, it captures both the limiting instances; viz. where TCA cycle can function for the last time (just before dying), and, on the other hand, how close to a functioning TCA cycle can the physico-chemical universe come.

Our approach towards the description of synchronization profile emerging out of biochemical pathways, is not in contradiction with the existing framework of studies on the same system from game theoretic perspective (typically the description from 'dynamic fitness landscape'[9]). The very fact that entropies in our approach were represented with respective mixed strategies, could implicitly take into account the 'dynamic fitness' all along. Since reliable data for kinetic parameters [18] for every variable for all the snapshots were available with us, the time-dependent and context-dependent nature of continuous adaptation scheme can be described with the present scheme in a reliable manner.

Methodology :

1) The mathematical backbone :

In order to model the entire situation as objectively as possible, an appropriate framework to describe the antagonistic nature of entropic clash was required. Since an actual conflict can be modeled by a finite non-cooperative game, we have used it in our study. We can justify the applicability of this construct, because a finite antagonistic game has the following characteristics :

1) The conflict is defined by the non-cooperative behavior of two sides, each of which can choose from a finite set of feasible actions (strategies). In the present case, entropies can choose strategies of their own to either minimize and maximize the profiles.
2) Each side estimates for itself. In other words, both sides choose their strategies independently of their adversary; which implies, each side has no preplay information on the actions of the other side; which obviously is extremely pertinent in our case.
3) The results of these actions is available in the forms of real numbers, which indicate unambiguously the utility of the strategy set for each side. The efficiency of any set of strategies for any side can be evaluated from the result of the interactions between two opponent strategies.
4) Results obtained from the actions of each sides (reflected in the time-dependent fluctuations in the concentration of any macromolecule, when the concentration is subjected to two opposing strategies and embodies the resultant effect of two opposite strategies) are inseparable and unique and therefore might be viewed as being the elements of some abstract sets, differing by the degree of efficiencies (utilities) that the

effect of strategies bring about.

Simplest mathematical description of such a mathematical game can be provided as :

$$\Gamma =< A, B, H > \tag{1}$$

where A and B denote the sets of possible actions of $SIDE-1$ and $SIDE-2$, respectively; and H denotes the utility function for $SIDE-1$ defined on the set $A \times B$.

Hence, in the parlance of game-theoretical studies, A forms the set of 'pure strategies' of $SIDE-1$ (*negative entropy*), while B represents the set of 'pure strategies' of $SIDE-2$ (*positive entropy*) ; while H is pay-off function for $SIDE-1$. We assume that only the principal actions from both $SIDE-1$ and $SIDE-2$ are considered for the game, so that function H is found adequately over the principal actions that define their conflict.

Since, in the context of our problem concerning concentration profiling of macromolecules in a system of biochemical pathways, the number of actions (strategies) is finite. Thus the stages of evolution of zero-sum finite game from the perspective of $SIDE-1$ (*negative entropy*), can be described by the matrix :

$$\mathbf{A} = \begin{bmatrix} a_{11} & a_{12} & \dots & a_{1n} \\ a_{21} & a_{22} & \dots & a_{2n} \\ \dots & \dots & \dots & \dots \\ a_{m1} & a_{m2} & \dots & a_{mn} \end{bmatrix} \tag{2}$$

Published data [18] for concentrations of 12 metabolites for the first 100 minutes of evolution of TCA cycle was reliably considered as the pure strategy. Since a TCA cycle does not perform its function in free space, but in conjunction with other pathways within a living biological cell; evolution of its concentration profile over the first 100 minutes provides an ideal framework to monitor the pure strategy of the player 'negative entropy'.

Description of $SIDE-2$ (*positive entropy*), is far less complicated. Since the objective was to model the entropic clash between emerging synchronization profile from actions denoting 'negative entropy' with the general depiction of randomness prevalent in the strategies and actions of 'positive entropy', we attempted the to capture the true disordered profile of entities in gaseous state by representing them in the same framework of 12×100 matrix (as the SIDE-1 matrix), where all the entries were random numbers. We assumed that the probability of a synchronization profile emerging out of a set of random numbers (generated from 'rand function' of the standard software MATLAB) is zero, for all practical purposes. However, to ensure a rapid convergence of our algorithm (by negating the scope of absurd comparability) we chose to scale up the MATLAB generated random numbers in the same range of concentrations by multiplying each one of the SIDE-2 entries (originally generated between 0 and 1) by the constant factor $\left[\left([a_{ij}]_{max} - [a_{ij}]_{min}\right) \forall i, j \quad A = \{a_{ij}\}\right]$. This causes no loss to generalization, yet assures a speedy convergence of the problem to the equilibrium condition. Hence, we formally define the framework to monitor the pure strategy of the player 'positive entropy' :

$$\mathbf{B} = \begin{bmatrix} b_{11} & b_{12} & \dots & b_{1n} \\ b_{21} & b_{22} & \dots & b_{2n} \\ \dots & \dots & \dots & \dots \\ b_{m1} & b_{m2} & \dots & b_{mn} \end{bmatrix} \tag{3}$$

In our pursuit, to describe the evolution of the game with as much truth as possible, for every matrix $\mathbf{A}$ (generated in the paradigm of negative entropy), corresponding $\mathbf{B}$ matrix (in the paradigm of positive entropy) is created.

Number m and n in matrices $\mathbf{A}$ and $\mathbf{B}$, describe of pure strategies of (*negative entropy*) and (*positive entropy*), respectively. For the biologists, any particular element, say a_{ij} for the matrix $\mathbf{A}$, denotes the concentration of some macromolecule in matrix $\mathbf{A}$; whereas for the game-theorists it implies the payoff of (*negative entropy*) in any situation, when being subjected to strategies i, j. The payoff matrix $\mathbf{A}$ represents, essentially, a game model of actual conflicts consistent with the four aforementioned conditions.

Our idea was to let these matrices interact; in other words, to let these players play the game. We were interested to systematically observe the approach of these two matrices to the Nash equilibrium.

Formally, we propose to model this situation with a two-person non-cooperative finite game $\mathbf{G_{AB}}$, where there are two $m \times n$ payoff matrices $\mathbf{A}$ and $\mathbf{B}$, defined for all pairs of strategies and corresponding to the payoffs for $SIDE-1$ and $SIDE-2$, respectively.

An equilibrium situation (captured by the pair of strategies ($\mathbf{X}^*$, $\mathbf{Y}^*$)) for this bi-matrix game exists if :

$$\left(\mathbf{X}^*\mathbf{A}\mathbf{Y}^{*\mathbf{T}}\right) \geq \mathbf{A_{i\rightarrow}}\mathbf{Y}^{*\mathbf{T}}, \qquad i = 1, 2, \dots, m \tag{4}$$

$$\left(\mathbf{X}^*\mathbf{B}\mathbf{Y}^{*\mathbf{T}}\right) \geq \mathbf{X}^*\mathbf{B_{\rightarrow j}}, \qquad j = 1, 2, \dots, n \tag{5}$$

Here $\mathbf{X}^* \geq 0$ and $\mathbf{Y}^* \geq 0$ are optimal strategies which ensure payoffs to both adversaries; $\mathbf{B_{\rightarrow j}}$ denotes j^{th} row of the matrix $\mathbf{B}$; similarly $\mathbf{A_{i\rightarrow}}$ denotes i^{th} row of the matrix $\mathbf{A}$; whereby, to summarize the framework we can write :

$$\left(\mathbf{X}^*\mathbf{E}_m^T\right) = \left(\mathbf{E}_n\mathbf{Y}^{*\mathbf{T}}\right) = 1\,, \quad \mathbf{E}_p = (1, 1, \dots, 1) \in R^p \tag{6}$$

For easy implementation with computational constructs, we transform these equilibrium conditions with the use of an $m \times n$ matrix $\mathbf{E}_{m,n}$ in which all entries are unities, and an arbitrarily chosen number k, which exceeds all the entries in matrices $\mathbf{A}$ and $\mathbf{B}$. Under such transformation, the inequalities will form the following set :

$$\mathbf{X}\left(k\mathbf{E}_{m,n} - \mathbf{B}\right) \geq \mathbf{E}_n^T,\ \mathbf{X} \geq 0 \tag{7}$$

$$\left[\mathbf{X}\left(k\mathbf{E}_{m,n} - \mathbf{B}\right) - \mathbf{E}_n^T\right]\mathbf{Y}^T = 0 \tag{8}$$

$$\left(k\mathbf{E}_{m,n} - \mathbf{A}\right)\mathbf{Y}^T \geq \mathbf{E}_m^T,\ \mathbf{Y} \geq 0 \tag{9}$$

$$\mathbf{X}\left[\left(k\mathbf{E}_{m,n} - \mathbf{A}\right)\mathbf{Y}^T - \mathbf{E}_m^T\right] = 0 \tag{10}$$

If a pair ($\mathbf{X}$, $\mathbf{Y}$) satisfies the equation set (7-10); then with with little replacement :

$$\mathbf{X}^{*}= \mathbf{X}/\mathbf{X}\mathbf{E}_{m}^{T}\,,\mathbf{Y}^{*}=\mathbf{Y}/\mathbf{E}_{n}\mathbf{Y}^{\mathbf{T}} \tag{11}$$

and observation of $eq^{n}-7$, we obtain :

$$\mathbf{X}^{*}\mathbf{B}_{\rightarrow \mathbf{j}}\leq k-1/\mathbf{X}\mathbf{E}_{m}^{T} \tag{12}$$

while in view of $eq^{n}-8$, we obtain :

$$\mathbf{X}^{*}\mathbf{B}\mathbf{Y}^{*\mathbf{T}}=k-1/\mathbf{X}\mathbf{E}_{m}^{T} \tag{13}$$

These last two conditions yield $eq^{n}-5$; similarly $eq^{n}-8$ and $eq^{n}-9$ yield $eq^{n}-4$.

Hence in a nutshell, the equilibrium situation ($\mathbf{X}^{*}$, $\mathbf{Y}^{*}$) defined by $eq^{n}-11$, is the solution of bi-matrix game (i.e., it satisfies $eq^{n}-4$ to $eq^{n}-6$). Conversely, if ($\mathbf{X}^{*}$, $\mathbf{Y}^{*}$) is a solution of $eq^{n}-4$ to $eq^{n}-6$, and if k is a certain positive number, then letting :

$$\mathbf{X}\mathbf{E}_{m}^{T}=\left(k-\mathbf{X}^{*}\mathbf{B}\mathbf{Y}^{*\mathbf{T}}\right)^{-1} \tag{14}$$

$$\mathbf{E}_{n}\mathbf{Y}^{\mathbf{T}}=\left(k-\mathbf{X}^{*}\mathbf{A}\mathbf{Y}^{*\mathbf{T}}\right)^{-1} \tag{15}$$

We can extend this representation to describe a holistic framework too. This can be easily achieved by observing that ($k\mathbf{E}_{m,n}-\mathbf{A}$) and ($k\mathbf{E}_{m,n}-\mathbf{B}$) may be arbitrary matrices with positive entries. Denoting them by $\mathbf{P}$ and $\mathbf{Q}$, we can describe the equation set (7-10) in the form :

$$\mathbf{X}\mathbf{Q}\geq \mathbf{E}_{n}\,,\ \mathbf{X}\geq 0 \tag{16}$$

$$\left(\mathbf{X}\mathbf{Q}\geq \mathbf{E}_{n}\right)\mathbf{Y}^{\mathbf{T}}=0 \tag{17}$$

$$\mathbf{P}\mathbf{Y}^{\mathbf{T}}\geq \mathbf{E}_{m}^{T}\,,\ \mathbf{Y}\geq 0 \tag{18}$$

$$\mathbf{X}\left(\mathbf{P}\mathbf{Y}^{\mathbf{T}}\geq \mathbf{E}_{m}^{T}\right)=0 \tag{19}$$

The set of vectors $\mathbf{X}$ satisfying equation set (16-19) form a complex polyhedron $\mathbf{U}$ bounded by $m+n$ hyperplanes.

2) The algorithmic implementation :

2.1) Background :

We start by explaining our approach about modeling the pure strategies of the players, viz. (positive entropy) and (negative entropy) in algorithmic and philosophical details .

The kinetic data (for the first 100 minutes of the TCA cycle) was taken from a computational study based on experimental evidences [18]. Since there are 12 metabolites in TCA cycle, such data provided us with a monolithic 12×100 matrix. This matrix was segmented into 88 12×12 overlapping square matrices, to obtain a stage-by-stage description of the synchronization process and to ensure computational ease, with no loss of generality of description. These 88 overlapping matrices described the evolution of strategies of negative entropy. We named this set of matrices (describing negative entropy) as **A**. As described in the 'introduction' section, correspondingly, 88 overlapping matrices were created to describe the evolution of strategies of positive entropy. We named this set of matrices (describing negative entropy) as **B**.

At this point, it assumes importance to clarify why the composition of matrix **B** was deliberately kept as purely random. This assumes significance because one might argue that identification of 'edge of life' in the context of the present problem will merely provide us with the 'edge of life' of TCA cycle. While there might be some ground behind such criticism, it is ill-directed and myopic in nature. Because, the principal objective of the present work was to describe neither the tolerance nor the efficiency of TCA cycle, but to quantify the order (negative entropy) hidden in the (time-dependent and context-dependent) synchronization profile of the concentrations of the metabolites involved in the TCA cycle. An unambiguous measure of the same could only be found if the TCA cycle is taken out of the negative entropic environment, already provided to it by the living biological cell and be exposed to physico-chemical universe directly. Such an operation is impossible to conduct in laboratory, with the present equipmental set-ups and contemporary knowledge of Biology.

However, history of science is gilded with $'gedanken\ experiments'$ that had accounted for huge success stories in different spheres of science over the years. Enormous impacts of $'gedanken\ experiments'$ like Maxwell's demon (thermodynamics), Schrodinger's cat(quantum mechanics), EPR paradox(quantum mechanics), twin paradox(special theory of relativity) - in the growth of Physics can never be underestimated. Although Biology is not so blessed with them, here we propose a $'gedanken\ experiment'$ that numerically counts the magnitude of negative entropy with respect to entropy of physico-chemical universe.

The distinct advantages of our work are twofold -
One, given the concentration profile for all the macromolecules in a biological cell; we can expand the present algorithm *as it is* to quantify how much of negative entropy does a particular cell (taken from any particular tissue of any particular organ etc..) embody.
Two, the entropic profile of any pathway (not only the TCA cycle) within a biologically functional cell can be obtained easily by simply implementing our algorithm with a suitable composition of **A** and **B** matrix.

Hence, it follows naturally that characterization of entropic profile of TCA cycle in a living cell is merely a special case of the general framework that is being suggested

in the present work. In particular, since the concentration profile of the constituents of a living cell already exemplify order (negative entropy) in several levels of organization, attempts to measure negative entropy in TCA cycle with respect to already existing (but unquantifiable) negative entropy of a cell, would have been a qualitative, drab, yet extremely difficult proposition. We opted for studying the general case of comparing negative entropy of a quintessential biological structure (the TCA cycle) with respect to the positive entropy of a comparable system from physico-chemical universe. As the results of this work proves, such comparison has provided us with rich (and unpredictable) set of information about Nature's scheme to ensure biological robustness. Furthermore, such comparison could quantify every biological quantity and/or property, it talked about.

2.2) Algorithm Execution :

Based on the mathematical framework and philosophical orientation of the aforementioned questions, our algorithm execution was multifaceted; although conforming always to the background elaborated above. Our approach comprised of :

2.2.1) Obtaining time variation data and playing the game

Since a thorough knowledge about the profile of pure strategy of players was necessary for us to understand their nature, determinants of all the 88 **A** and **B** matrices were calculated. Such analysis had provided us with the time variation data of behavior of TCA cycle for the matrix **A** and a profile of the time variation of concentration of random variables (scaled w.r.t **A**, as had been mentioned earlier during construction of matrix **B**) as described by **B**.

To observe the interplay of positive and negative entropy on the concentrations of the metabolites, we resorted to make the players play by the mixed strategy. Here to simulate the effect of the game, negative entropy of the **A** matrix was disrupted by the introduction of positive entropy in it. These tiny quanta's of positive entropy were named 'perturbations'. Out of innumerable possible ways of adding perturbations to the elements of **A**, we had employed three. A brief description and small comparative studies of these three is given below, because certain perturbation strategies might find potent use in certain cases.

2.2.1.1)Perturbations to elements of A : strategy 1

In the (easily implementable) arbitrary strategy of introducing perturbation; using a variable n as the pointer to the columns(concentrations of individual metabolites) of the matrix starting from $n = 0$, we added (fictitious) concentrations to every $(2n+1)^{th}$ element of every row (snapshot of all the concentrations at any given instance of time) for all the 88 matrices. Just to maintain the symmetry of the situation, and to increase the positive entropy by allowing the system to have more disparity, starting from $n = 1$, we subtracted (fictitious) concentrations from every $(2n)^{th}$ element of every row for all the 88 matrices.

However, such addition and subtraction of (fictitious) concentrations were completely arbitrary and although it could perturb the system sufficiently, the amount of (fictitious) concentrations added to or subtracted from - could have always assumed an unstructured arbitrary nature; which in turn, would have made it difficult to implement computationally with an efficient algorithm.To avoid such situation, we attempted to introduce the perturbations from a more structured and consistently implementable approach, details of which are as follows.

2.2.1.2) Perturbations to elements of A : strategy 2

Here, to start with, the metabolites are enumerated in an increasing order by sorting the magnitude of concentrations. Then a systemic perturbation was introduced in a single shot by adding a factor ($\sim$50000000) to the concentration of six lowest concentrations; whereas the same factor was subtracted from six concentrations with highest magnitudes.

However, the exact choice of the perturbating factor might be biologically insensitive (not all the concentration profiles are equally perturbed by the same perturbating factor, one that is too high for someone, might well be accounting for negligible effects in another; etc ..). Thus we resorted finally to a scheme of proportional perturbation, where the sorted magnitude of concentrations were scaled up or scaled down by a factor of the presence of its own.

2.2.1.3) Perturbations to elements of A : strategy 3

In the final analysis, 12 metabolites were sorted with respect to the magnitude of concentration, at the initial time $t = 1$. From these 12, the first three were identified as the boundary metabolites. They were kept constant throughout the course unless system was not at steady state. Concentrations of the 4 metabolites with low magnitudes were increased by the 0.5 of their own proportion at that instance, while concentrations of the 5 metabolites having high magnitudes were decreased by the 0.5 of their proportion at that time instance.
Advantage of implementing this proportional perturbation, is multidimensional. It bring a rationality (and therefore implementable structure) in the process of introducing positive entropy in the system. Even more importantly, in the realm of sensitivity analysis, an unambiguous pattern of any particular macromolecule's capability of affecting the entire system of synchronized concentrations - can be found by suitable application of it.

The **B** matrix was also subjected to the mixed strategy as we wanted to study how close a randomly chosen structure, from physico-chemical universe, can come to a functioning biological system. Since every **B** was generated randomly (within the range of the corresponding **A**), probability of existence of any synchronization profile in it was abysmally small. Quite expectedly, **B** contained enormous amount of entropy as compared to **A** (as confirmed by the profiles of pure strategy). However, unlike the case of matrix-**A**, where we were pumping positive entropy into, from **B**, we sucked positive

entropy out. In the parlance of mixed strategy, this was equivalently described as pumping negative entropy into **B**. This was achieved in a step-by-step manner where we first calculated if $([b_{ij}] > [a_{ij}])$ is positive or negative.

2.2.1.4) Perturbations to elements of B : strategy 1

If $([b_{ij}] > [a_{ij}])$ is positive , $[b_{ij}] = [b_{ij}] - \frac{([b_{ij}]-[a_{ij}])}{2}$ was implemented.

2.2.1.5) Perturbations to elements of B : strategy 2

If $([b_{ij}] > [a_{ij}])$ is negative $[b_{ij}] = [b_{ij}] + \frac{([b_{ij}]-[a_{ij}])}{2}$ was assigned.

2.2.1.4 and 2.2.1.5 was implemented in an iterative manner. It is easy to notice that merely subtracting the (fictitious) concentrations from **B** will not account for the reduction in its entropy profile, but only bring the (a)synchronization-profile to a lower magnitude.

Having established the perturbation scheme, we studied the non-satisfiablity of the inequalities $\mathbf{A} \geq \mathbf{A_{i\rightarrow}}$ and $\mathbf{B} \geq \mathbf{B_{\rightarrow j}}$, from the first approach. An exhaustive analysis of enormous biological significance was performed in a systematic manner, where, to incorporate the mixed strategy, perturbations were incorporated in every step to observe the non-satisfiablity of the inequalities mentioned above. The detailed algorithm to implement this process is given in section 2.2.

2.2.2) Implementation of mathematics from two different perspectives :

Although the mathematical framework suggested beforehand provides an elaborate scheme of describing the situation, - the crux of the model boils down to the two most basic equations therein; namely $eq^n - 4$ and $eq^n - 5$. Indeed it was shown in the beforehand that while $eq^n - 12$ and $eq^n - 13$ yield $eq^n - 5$, $eq^n - 8$ and $eq^n - 9$ produce $eq^n - 4$. Thus, a primary (yet most reliable) investigation of the system dynamics is performed in the present study, with a thorough implementation of $eq^n - 4$ and $eq^n - 5$; although a large-scale work with more detailed characterization of variables might call for the implementation of other equations too.
Both $eq^n - 4$ and $eq^n - 5$ depend as heavily on the characteristics of matrices **A** and **B** as on the vectors **X** and **Y**. However, while **A** and **B** are comprised of numerical variables, the typical description of vector **X** will contain character variables, viz., the names of metabolites (for vector **Y**, this problem will be trivial, but still, existent nevertheless). Hence, to deal with this implementational difficulty, we approached the problem from two viewpoints that are fundamentally different from each other in their motivation and implementational complexity.

2.2.2.1) First approach

Here the information regarding which metabolite is represented by how much concentration, in a functioning TCA cycle, is considered.

The problem mentioned beforehand was redefined by trivializing the character valued vectors; whereby the conditions of $eq^n - 4$ and $eq^n - 5$, to detect the equilibrium (in other words, the EOL of TCA cycle) were reduced to detection of cases where $\mathbf{A} \geq \mathbf{A_{i\rightarrow}}$ and $\mathbf{B} \geq \mathbf{B_{\rightarrow j}}$ - are observed. The magnitude of $\mathbf{A}$ was given by the magnitude of determinant of anyone of 88 (12×12) matrices, keeping a balance sheet of concentrations of all the 12 metabolites flowing in and flowing out of the TCA cycle for any continuous stretch of 12 minutes. $\mathbf{A_{i\rightarrow}}$, in such a description naturally refers to a row vector; viz. concentration of 12 metabolites at any frozen instance of time. (Similar logic applies for $\mathbf{B}$, and $\mathbf{B_{\rightarrow j}}$). This made Biological sense, especially during the observation of the extent of change in the magnitude of determinant of $\mathbf{A}$, when perturbations were applied (during mixed strategy implementation) on a single time instance across all the 12 metabolites.

Details of the algorithm are as follows.

Algorithm 1: Approach to the Edge of Life for A MATRIX and B MATRIX

1 : FOR THE FIRST 12×12 MATRIX A [OR B]

(FIRST MATRIX ACCOUNTS FOR 1st MINUTE TO 12th MINUTE)

1.1:
CALCULATE ABSOLUTE VALUE OF DETERMINANT OF THE MATRIX, ASSIGN IT TO A [OR B].

1.2:
CALCULATE THE ABSOLUTE VALUE OF EVERY ROW (VECTOR) OF THE MATRIX ASSIGN IN Ai [OR Bi]

1.3:
SAVE THESE Ai [OR Bi] FROM ALL THE ROW-VECTOR-MAGNITUDES IN A NEW MATRIX.

1.1.1 :
FOR FIRST ROW

1.1.1.1 :
CALCULATE THE ABSOLUTE VALUE OF DETERMINANT OF THE ROW (VECTOR), ASSIGN IT TO Ai1 [OR Bi1]

1.1.1.2 :
PERTURB ONE ROW AT A TIME.

FIND OUT THE LOWEST VALUE OF Ai/Bi FOR WHICH (A < Ai) [OR (B<Bi)]

ASSIGN [i-1] TO 'EDGE_1-12_ROW_1_MAGNITUDE'

1.1.2 :
COME OUT OF THE FIRST ROW

1.4 :
REPLACE BACK THE ORIGINAL FIRST ROW IN THE MATRIX

1.2.1 :
GET INTO THE SECOND ROW

1.2.1.1 :
CALCULATE THE ABSOLUTE VALUE OF

DETERMINANT OF THE ROW(VECTOR), ASSIGN IT TO Ai2 [OR Bi2]

1.2.1.2 :
FIND OUT THE LOWEST VALUE OF Ai FOR WHICH (A < Ai) [OR B < Bi]
ASSIGN [i-1] TO 'EDGE_1-12_ROW_2_MAGNITUDE'

1.2.2 :
COME OUT OF THE SECOND ROW

1.5 :
REPLACE BACK THE ORIGINAL SECOND ROW IN THE MATRIX

1.3.1 :
GET INTO THE THIRD ROW · · · · · · TILL 12TH ROW:

COME OUT OF THE FIRST MATRIX.

2 : GET INTO THE SECOND 12×12 MATRIX
(SECOND MATRIX ACCOUNTS FOR 2nd MINUTE TO 13th MINUTE)
CARRY ON WITH THE SAME SET OF OPERATIONS AS WITH THE FIRST MATRIX.
COME OUT OF THE SECOND MATRIX.

3 : GET INTO THE THIRD 12×12 MATRIX
(THIRD MATRIX ACCOUNTS FOR 3rd MINUTE TO 14th MINUTE)
CARRY ON WITH THE SAME SET OF OPERATIONS AS WITH THE FIRST MATRIX.
.
.
.
88 : GET INTO THE 88th 12×12 MATRIX
(88th MATRIX ACCOUNTS FOR 88th MINUTE TO 100th MINUTE)
CARRY ON WITH THE SAME SET OF OPERATIONS AS WITH THE FIRST MATRIX.

2.2.2.2) Second Approach

In this case, we count how many metabolites are there in a functional TCA cycle within a given range of concentration. The entire spectrum of active concentrations of the participating metabolites is divided with the total number of metabolites, in order to get range of concentration.

Here, since it was not imperative to know which metabolite was represented by how much concentration (a necessary biological question), magnitude of $\mathbf{A}$, $\mathbf{B}$, $\mathbf{X}$, $\mathbf{Y}$, $\mathbf{X}^T$ and $\mathbf{Y}^T$ - could all be calculated with precise numerical magnitudes. Dividing the spectrum of concentrations by 12, 12 bins of concentrations were constructed; each one of which was representing a range of concentration of functional metabolites involved in the TCA cycle. While that stood for $\mathbf{A}$; $\mathbf{X}$ represented the number of metabolites with a given range of concentration; and finally, $\mathbf{X}^T$, denoted the transpose of the matrix $\mathbf{X}$. (Accordingly $\mathbf{B}$, $\mathbf{Y}$ and $\mathbf{Y}^T$ could be represented too). It is not difficult to observe that everyone of $\mathbf{A}$, $\mathbf{B}$, $\mathbf{X}$, $\mathbf{Y}$, $\mathbf{X}^T$ and $\mathbf{Y}^T$, in the second approach, could be reduced to vectors without compromising with the mathematical rigor of description of the situation. Further, it can hardly go unnoticed that if the number of metabolites in the TCA cycle was more than 32 (the parametric threshold), the possibility of observing a power-law type distribution, at least in the matrix $\mathbf{A}$, could very well be contemplated too; - however such possibility, obviously could not arise in the present case.

Details of this approach are as follows.

Algorithm 2: Approach to the Edge of Life for A MATRIX and B MATRIX

1: FOR THE FIRST 12×12 A MATRIX

(FIRST MATRIX ACCOUNTS FOR 1st MINUTE TO 12th MINUTE)

1.1:
CALCULATE ABSOLUTE VALUE OF DETERMINANT OF MATRIX, ASSIGN IT TO A.

1.2:
CALCULATE THE ABSOLUTE VALUE OF EVERY ROW (VECTOR) OF THE MATRIX
ASSIGN IT TO Ai.

1.3:
CALCULATE THE RANGE OF CONCENTRATION OF EACH ROW.

1.4
CREATE THE 12 BINS OF INTERNALS OF RANGE. ASSIGN IT TO ROW
MATRIX X.

1.5
CLASSIFY THE METABOLITES WITH RESPECT TO THEIR CONCENTRATION
IN MATRIX A.

CALCULATE IN EACH BINS HOW MANY METABOLITES ARE THERE.

1.6 REPEAT THIS PROCEDURE FOR EACH Ai.

2: FOR THE FIRST 12×12 A MATRIX B

(FIRST MATRIX ACCOUNTS FOR 1st MINUTE TO 12th MINUTE)
1.1:
CALCULATE ABSOLUTE VALUE OF DETERMINANT OF MATRIX, ASSIGN IT TO A.

1.2:
CALCULATE ABSOLUTE VALUE OF EVERY ROW (VECTOR) OF MATRIX ASSIGN IT TO Ai.

1.3:

CALCULATE THE RANGE OF CONCENTRATION OF EACH ROW.

1.4
CREATE THE 12 BINS OF INTERVALS OF RANGE. ASSIGN IT TO COLUMN MATRIX Bj.

1.5
CLASSIFY METABOLITES WITH RESPECT TO THEIR CONCENTRATION IN MATRIX A.

CALCULATE IN EACH BINS HOW METABOLITES ARE THERE.

1.6 REPEAT THIS PROCEDURE FOR EACH Bj.

3. SOLVE FOR THE EQUATION XAYt >= Ai Yt.

3.1 PERTURB THE X AND Y, TILL XAYt <Ai Yt

3.1.1. PERTURBATION METHODOLOGY:
CALCULATE THE DIFFERENCE BETWEEN X AND Y CORRESPONDING ELEMENTS. IF THE DIFFERENCE IS POSITIVE, THEN ADD THE DIFFERENCE TO X AND SUBTRACT IT FROM Y.

4. REPEAT STEP 3 FOR EACH ROW OF THE MATRIX.
.
.
.

5. REPEAT ENTIRE PROTOCOL (STEP 1-4) FOR 88 MATRICES

This implies, the biological information of associating a particular magnitude of concentration with a particular metabolite is not respected in the second approach. But the second approach could provide us with an inherent advantage that the first couldn't; namely, the description of $eq^n - 4$ and $eq^n - 5$, could all be done with numerical values of $\mathbf{A}$, $\mathbf{B}$, $\mathbf{X}$, $\mathbf{Y}$, $\mathbf{X}^T$ and $\mathbf{Y}^T$.

Thus, while the first approach was easily relatable with biological experience,

the second one was mathematically more correct. However, since both of them were providing unique advantages, it was not possible for us to ignore anyone of them.

3) Sensitivity Analysis :

We made sure that in every instance when an entry is changed (that is, magnitude of that particular concentration is changed), rest of the concentrations are not perturbed from their biologically functional level of magnitude. A random magnitude was substituted as the modified concentration for each of these entities (one-at-a-time scheme). Actual biological analogue for such change could be mapped to events like that of gene knockouts [15], change in a substrate like a carbon source, or the addition of a protein inhibitor like a drug, or anything else. Actually, for the particular question we are discussing in this work, such mapping may be considered unwarranted as well.
The detailed algorithm to implement this process is as follows.

Algorithm 3: Approach to the Edge of Life and sensitivity of each metabolite :

1: FOR THE FIRST 12×12 MATRIX

(FIRST MATRIX ACCOUNTS FOR 1st MINUTE TO 12th MINUTE)

1.1:
CALCULATE ABSOLUTE VALUE OF DETERMINANT OF THE MATRIX, ASSIGN IT TO A.

1.2:
CALCULATE THE ABSOLUTE VALUE OF EVERY ROW (VECTOR) OF THE MATRIX ASSIGN IN Ai

1.3:
SAVE THESE Ai FROM ALL THE ROW-VECTOR-MAGNITUDES IN A NEW MATRIX.

1.1.1 :
FOR FIRST ROW METABOLITE 1

1.1.1.1 :
CALCULATE THE ABSOLUTE VALUE OF DETERMINANT OF THE ROW (VECTOR), ASSIGN IT TO Ai1

1.1.1.2 :
PERTURB ONLY ONE METABOLITE AT A TIME WHILE KEEPING THE REST OF THE ROW INTACT.
FIND OUT THE LOWEST VALUE OF Ai FOR WHICH (A < Ai)
ASSIGN [i-1] TO 'EDGE_1-12_ROW_1_MAGNITUDE'

1.1.2 :
COME OUT OF THE FIRST ROW

1.4 :

REPLACE BACK THE ORIGINAL FIRST ROW IN THE MATRIX
FOR FIRST ROW METABOLITE 2. 12

1.1.1.3 :
CALCULATE THE ABSOLUTE VALUE OF

DETERMINANT OF THE ROW (VECTOR), ASSIGN IT TO Ai2

1.1.1.4 :
PERTURB ONLY ONE METABOLITE AT A TIME WHILE KEEPING THE REST OF THE ROW INTACT.
FIND OUT THE LOWEST VALUE OF Ai FOR WHICH (A $<$ Ai)

ASSIGN [i-1] TO 'EDGE_1-12_ROW_1_MAGNITUDE'

1.1.3:
COME OUT OF THE FIRST ROW
1.2.1 :
GET INTO THE SECOND ROW. . . . . .12 ROW

2: GET INTO THE SECOND 12×12 MATRIX. . . . . .88 MATRIX

Results and Discussions :

1)Results obtained from game with pure strategies :

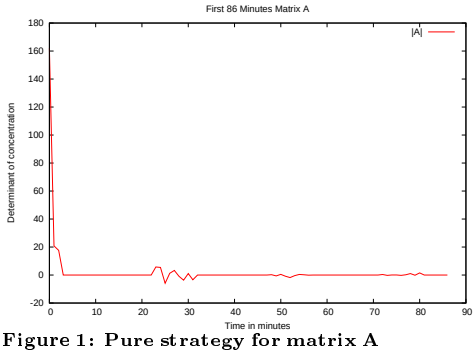

Figure 1: Pure strategy for matrix A

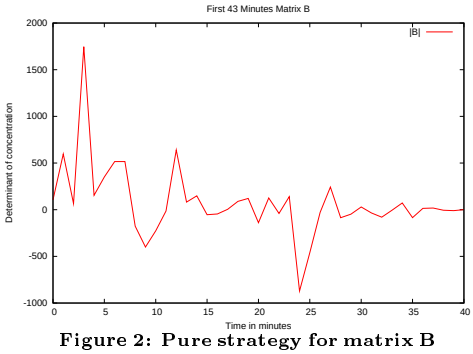

Figure 2: Pure strategy for matrix B

Results of evolution of **A** depicts the behavior of TCA cycle while trying to achieve steady state with time evolution. Prominent biological facts from this graph are manifold. They are :

a) System is at non-steady during $t = 0$ to $t = 3$ (time in minutes). One can however, notice from the trend of the plot (Fig: 1) that, system is attempting to attain an asymptotic stability.
b) From $t = 4$ the system achieves steady state (determinant of $\mathbf{A} = 0$) and remains in it.
c) Although unexpected, some of the deviations from the steady state (determinant of $\mathbf{A} \neq 0$) were observed in the system namely at $t = 23$ to $t = 48$. Magnitude of determinant of **A** was small (< 5.00); nevertheless islands of instabilities were observed in two occasions. This finding was crucial and the implications of these seeds of instabilities helped us to understand certain (otherwise intractable) features during sensitivity analysis study.

For the matrix **B**, the expected pattern of absence of any tendency in the graph (Fig:2) was observed. Since **B** is comprised of purely random numbers, this was expected.

2) Results obtained from game with mixed strategies :

As indicated earlier, almost the entire extent of the present study was carried out with two parallel approaches; one, easily relatable to biological intuition; the other, mathematically more rigorous, but difficult to relate biologically to. Results for each of these approaches on all the considerations were obtained and are presented with (almost self-explanatory) series of graphs. Our findings could be grouped in two clusters; one, the unforeseen ones (like the plots of approach towards EOL, from negative entropic and positive entropic sides, respectively; along with associated ones); two, many a set of known results (some contemplated from theoretical study of TCA cycle energetics, some experimentally found results from particular cases of biological reality) were reproduced here, in which the complete transparency of simple mathematical and algorithmic constructs may allow the researchers to numerically describe their studies and generalize the particular trends.

We report all of the obtained results, irrespective of their belonging to anyone of these classes. An exhaustive analysis about the pattern recognition in disruption of negative entropy to EOL and positive entropy's attempt to touch the EOL, can meaningfully be done if both these sets of results (slew of plots provided herewith) are extensively studied. Since the entire body of obtained results from all the different versions of the algorithm is huge and mostly in the form of (almost) self-explanatory graphs; sketches of them are talked about in the following paragraphs.

As mentioned in the **Methodology** section, game with mixed strategy can be simulated by introducing positive entropy to TCA cycle (in the form of proportional perturbations), while pumping the negative entropy into the system having random profile.

We found that under the conditions that concentrations of the boundary metabolites (described in the first 3 columns of the matrix $\mathbf{A}$) are kept constant, even if the concentrations of the other metabolites are varied extensively, system tries to nullify these perturbations. It was observed that the system attempts to regain the steady state, at some other possible local minima (determinant of $\mathbf{A} = 0$) at the state-space of it, albeit with vastly different magnitude of metabolite concentrations. Such assertion in the paradigm of TCA cycle is not entirely new, but our approach vindicates that observation from a whole new perspective altogether.

It was found that the matrix $\mathbf{A}$ resides in such deep minima in its state-space that variants of perturbation strategies failed to inject enough entropy to make TCA cycle come out of its steady-state. Perturbations were indeed attempted with addition or subtraction of large magnitude of (fictitious) concentrations; but owing to these perturbations, as the magnitude of $\mathbf{A_i}$ increases, so does the magnitude of $\mathbf{A}$; and hence the algorithm fails to converge. Thus we could infer that with the change in the non-boundary metabolite concentrations, TCA cycle can adjust itself to an extent that it could not be perturbed; with all types of (aforementioned) perturbation strategies, and that too with multiples of 1000 iterations.

These results however were in stark contrast to the results obtained from applying perturbations to anyone of the boundary metabolites. To our astonishment, it was found out that these needed a perturbation, merely to the extent of 10^{-6}, to make the TCA cycle perturbed to the extent that the EOL (described crudely in the first approach, viz. satisfying the inequality $\mathbf{A} \geqslant \mathbf{A_i}$) could be observed. While this was known too; just like the previous finding, here also, our approach establishes the known fact from an all-different standpoint. It is obvious that the very fact of huge systemic scale effect upon a little change in concentration of any of the boundary metabolites can find wide-spread use in the realm of drug target specification and drug discovery.

An extremely interesting situation came to fore when proportionate perturbations were systematically applied to observe the effect on the matrix $\mathbf{A}$ with antagonistic trend of the same in $\mathbf{A}_i$; in particular with consistent trend of lowering the concentrations for all the 12 metabolites for 88 matrices. The (naive) biological intuition of ours had prompted us to predict that significant decrement of concentrations of the metabolite with biggest concentration alone, might cause the cell to reach the EOL.
Obtained results, on the other hand, suggested that if (due to perturbations) we let the magnitude of $\mathbf{A}_i$ to increase on the face of a systemic trend of decrease (due to perturbations) of $\mathbf{A}$; after some particular time instance (dependent on $'i'$) these values become constant and they stop from

approaching each other. In other words, the depth of synchronization profile (global minima of negative entropy in the state space in TCA cycle) was so deep that although the antagonistic perturbations could make the system unstable till an extent, these perturbations could not stretch the negative entropy to the extent that EOL is reached. From an utilitarian point of view, it implied that the proposed methodology (please refer to 'Algorithm 1') could quantify the extent of possible increase in the metabolite concentration, till which the system (viz. matrix $\mathbf{A}$) can tolerate antagonistic behavior of $\mathbf{A}_i$.

But like the plot of an eventful mystery story, it was found that EOL is reached the moment concentrations of metabolites were lowered. This result was significant. It makes eminent biological sense because a systemic trend of heterogeneous rate of lowering the concentrations of several metabolites will cause an accumulation of some(at least, one) metabolite in the cell. However, since the other metabolites, who could have accounted for this accumulation are systematically drained out, the TCA cycle could not sustain this attack on the negative entropy content of it. Hence, the cell was brought immediately to the EOL. While several (empirical and heuristic) analogies of this strategy are 'known', especially in the paradigm of drug designing, the uniqueness of the present methodology lies in measurement of the exact numerical extent of all the variables under consideration.

An elaborate (time-frame to time-frame) description of systems behavior (that is varying profile of determinant of $\mathbf{A}$) under the face of antagonistic perturbations (steadily perturbed profile of magnitude of row vector $\mathbf{A}_i$), is extremely interesting and rich source of biological information, studied with an incisive algorithm. Since we found the obtained set of data from such analysis can be considered independently as a coherent body of work, it has been kept separately in the section below.

2.1) Results from first approach
(Change in the profile of A under antagonistic perturbations from $\mathbf{A}_i$)

Profile of determinant of matrix $\mathbf{A}$ were showing monotonic behavior for most of the time instances. This is expected out of determinant of any matrix (containing macromolecular concentrations) in steady state. Hence it is not merely the profile of determinant of matrix $\mathbf{A}$, but the changes in the same, that described stages of interest and sources of new information. Therefore, here we are narrating only the time instances where magnitude of matrix $\mathbf{A}$ was showing tendencies to change from its then existing monotonic behavior.

As it turns out, the deviations from monotonic behavior in the profile of determinant of matrix $\mathbf{A}$, could be loosely be related to biological robustness studies.

2.1.1) During time instance t=1 and t=2 :

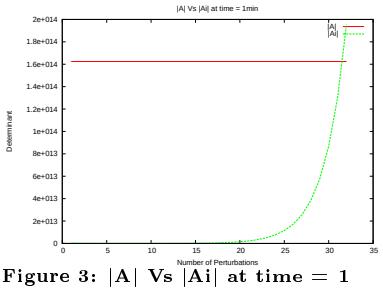

Figure 3: |A| Vs |Ai| at time = 1

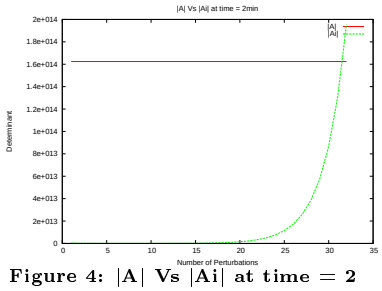

Figure 4: |A| Vs |Ai| at time = 2

As the concentration of every column (that is every individual metabolite, $[a_{i,\,j}]$) was proportionately perturbed, magnitude of $\mathbf{A}_i$ changed consistently; although magnitude of $\mathbf{A}$ remained invariant. This situation might apparently seem paradoxical, but a close inspection of correlation between this result and one obtained from COPASI, helps us to solve this paradox. The $(t = 1\ to\ t = 2)$ profile of determinant of $\mathbf{A}$, simply suggests that since the system (matrix $\mathbf{A}$) is asymptotically stable during present state of system's evolution; the state space is already showing convergent nature of Lyapunov coefficient and has already fallen in some local minima.
Hence, the determinants $\mathbf{A}_0$ and $\mathbf{A}_1$(who are nothing else than balance sheet of concentration profile of 12 metabolites over the first 13 (first to twelfth and second to thirteenth) minutes) are showing reluctance to come out of this local minima. The biological reason for this is equally simple to understand. Once $\mathbf{A}_0$ and $\mathbf{A}_1$ have found the local minima (a particular combination of concentration range of interacting metabolites which can offer a suitable template for the synchronization profile to emerge; there can be many of them), they will try to nullify any perturbation that would have attempted to disturb the synchronization profile. Or else the same phenomenon can be explained as, the perturbation applied on the system was not enough to change the already acquired negative entropic profile during the asymptotic stability that it has gathered during the first 13 minutes. It is not difficult to establish the equivalence between them.

The number of multiples of perturbations were observed to be 32 for $(t = 1\ to\ t = 2)$.

2.1.2) During time instance t=3 :

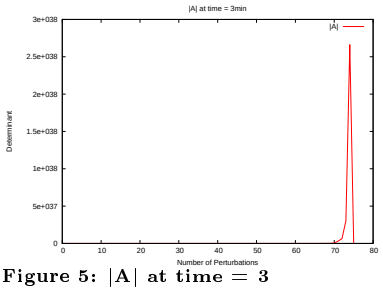

Figure 5: |A| at time = 3

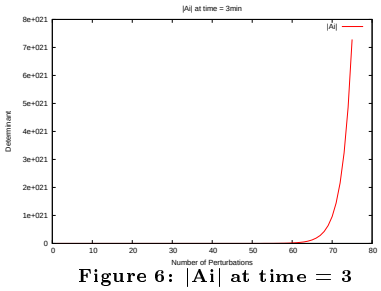

Figure 6: |Ai| at time = 3

At $t = 3$, the asymptotic stability of the system approaches a complete stability; hence the depth of the local minima of negative entropy deepens and it becomes difficult for the proportional perturbations applied to $\mathbf{A}_i$ to drag the TCA cycle out of its negative entropic groove (Fig: 5 & Fig: 6). This was unambiguously captured by the number of multiples of perturbations required in order to show the effect of perturbation on a particular row (anyone of the 12 concentrations at any time instance) on the whole matrix (concentration of 12 metabolites in TCA cycle from 3^{rd} minute to 14^{th} minute of its evolution). Magnitude of this number turned out to be more than the same in case of what was observed for $\mathbf{A}_0$ and $\mathbf{A}_1$. This implied that the robustness of the system is increasing, so that the tolerance profile of it against the increasing magnitude of perturbations is increasing too.

2.1.3) At steady state :

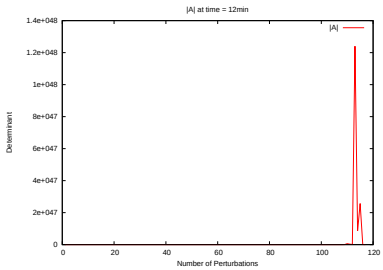

Figure 7: |A| at time = 12

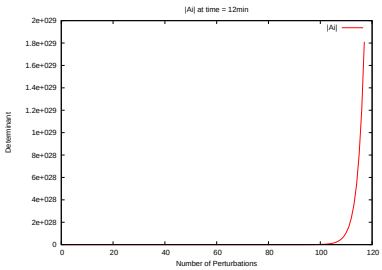

Figure 8: |Ai| at time = 12

As expected the tolerance of the TCA cycle negative entropy (embodied by the profile of determinant of $\mathbf{A}$) was found to be maximum at the steady state and it could account for the maximum perturbations at this stage (Fig: 7 & Fig: 8). We can generalize this finding to asset that the robustness of the system (it can be any other biochemical network too, the generalized nature of present algorithm ensures that the profile could not have been different in its case either) can be considered to be highest when it is at steady state.

2.1.4) At the edge of life for TCA cycle :

The maximum value of $\mathbf{A}$ for which it could tolerate the proportional perturbations in $\mathbf{A}_i$, in the inequality $\mathbf{A} \geqslant \mathbf{A_i}$ was termed as the Edge of Life (EOL) for TCA cycle.

Given this definition of EOL, with respect to every time instance and possible ranges of perturbations, there will be an EOL, when TCA cycle's inherent negative entropy could tolerate the perturbations. Thus the EOL could account for highest tolerance of the system under consideration and under the boundary conditions (time and nature of proportional perturbation applied to specific set of metabolites). However if the system is more perturbed at this point, it can not come back to steady state. From a game theoretic perspective, "Nash equilibrium" is achieved between positive entropy imparted on the system and negative entropy possessed by the system.

2.2) Results from second approach

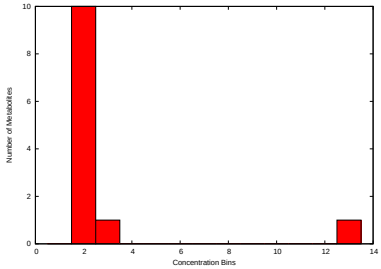

Figure 9: Before perturbation - Matrix A

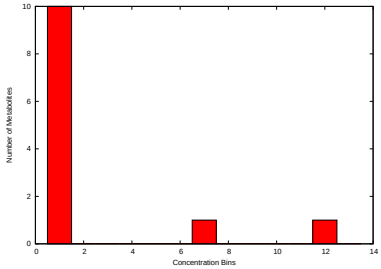

Figure 10: After perturbation - Matrix A

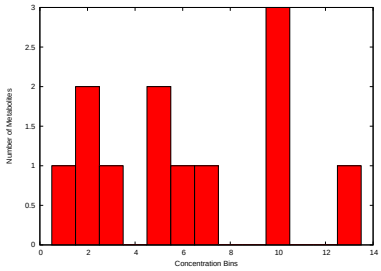

Figure 11: Before perturbation - Matrix B

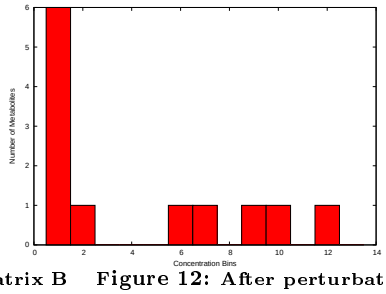

Figure 12: After perturbation - Matrix B

Study from second approach (where the onus was on the quantification of metabolites within binned ranges of concentration, as mentioned in the methodology section), yielded some unexpected results.

It was expected that 'number of metabolites' (the ordinate) versus 'range of concentration' (the abscissa) for the random system (described by changing profile of Matrix B) will show a normal distribution of metabolites at time $t = 0$ (Fig-11). As the negative entropy is introduced in the system, the aforementioned plot started to change in its profile and started to assume a one, in which a certain concentration range was showing increasing tendencies of being populated, instead of every bin being populated with similar probabilities. This was expected, because with the introduction of negative entropy, the random system started to assume a nature, more akin to those observed in

the Biological systems.

Finally, at the stage Bj is greater than B, the concentration range profile shows, what could have been called a 'power law'-type distribution (Fig-12), if the number of metabolites were more than the Gaussian parametric limit. Findings from the TCA cycle (described by changing profile of Matrix A) was expected to provide a trend, just mirror opposite to what was observed for B. However, to our profound astonishment, it turned out to be quite different. While the 'number of metabolites' (the ordinate) versus 'range of concentration' (the abscissa) analysis for A was started, the profile looked heavily skewed (Fig-9) as 10 out of 12 metabolites had their concentrations in a particular range of concentration (5.27×10^{10} to 1.05×10^{11})μM and only 2 could be observed to possess different concentration ranges than these 10. The truly startling feature of the concentration profile amongst TCA cycle metabolites came to be noticed when it was subjected to deliberate increase of positive entropy (in the form of perturbations). Even though the the magnitude of determinant A was becoming comparable to Ai, this heavily asymmetric pattern (10 in one concentration range, whereas 2 are scattered over 11 other possible ranges of concentration) persisted. Even at the point, where EOL of TCA cycle was being observed,the distribution of concentration profile of A did not waive (Fig-10), but had merely undergone a range transformation from (5.27×10^{10} to 1.05×10^{11})μM to less than $5.27\times10^{10}\mu$M.

This unflinching tendency of a biological system to have most of its components in one concentration range (even in the face of intense perturbation) might be one of the methodologies by which it provides itself with more opportunities to ensure a feasible platform for the interactions to take place, at the very first place. A disparate distribution of concentrations might not have been so conducive to ensure an even playing ground for Brownian collisions. The combinatorial possibilities of starting a scheme of interactions assumes a larger value if many (10 out of 12, in this case) metabolites possess concentrations in the same range; than what could have been the case if 'number of metabolites' versus 'range of concentration' plot was displaying a normal distribution (in such a case the shear number of combinatorial possibilities to construct a new scheme of reaction would have been less).

This finding was a revelation; purely because of the fact that biological systems maintain the possibility of starting a new series of interactions, even when perturbed to the edge of their lives, was unexpected. The steadiness of the 'number of metabolites' versus 'range of concentration' plot can as well be used to describe biological robustness from a new light. Although innumerable studies on biological robustness had thrown lights on several facets of it, understanding biological robustness from the (un)changing profile of 'number of metabolites' versus 'range of concentration' plot, when the system itself is being knocked over; can undoubtedly describe robustness in literal sense.

Results from the second approach, from this light, provides us with possibilities to construct systemic level markers that can describe biological robustness from the perspective of comparative scaling of concentrations of systemic components.

3) Results from Sensitivity Analysis :

Results from sensitivity analysis is kept below in a self-explanatory table.

Amongst the most prominent trends, it has been found that proportional perturbations applied on the concentration of the 12 metabolites can overtake the content of negative entropy embodied by the TCA cycle. In fact, since the system, described by the determinant of the matrix **A** was too sensitive (as mentioned beforehand) to the perturbations applied to the 3 boundary elements; taking them to consideration was not providing us with any new source of information; hence, the entire analysis was carried out on the other 9 metabolite concentrations. The response of the negative entropy (and hence robustness) of the system with respect to systematic proportional perturbations were not found from literature. In that respect the present analysis with sensitivity of the system assumes enormous importance.

Table kept below, is self-explanatory. However, a careful observation coupled with our previous findings reveal a deeper relationship between the nature of negative entropy and perturbations that the system can account for. The islands of small deviations from the ideal steady-state behavior was already reported beforehand. It has been found that while the system can rest without artificially imparted perturbations(fluctuations), it can tolerate these small deviations. But whenever anyone of these 9 variables could amplify these small seeds of instabilities, the negative entropy content of TCA cycle could not cope with it. Therefore, it is precisely at those instances that the system was becoming unstable.

Columns of the 'Sensitivity Analysis' table are containing minute-by-minute evolution data for the TCA cycle, whereas the rows are describing the (perturbed) concentration of the metabolites. The words **'able'** or **'unable'** denote the capability of the metabolite concerned, to perturb the system. The 5^{th} and 7^{th} column show peculiar behavior, in the sense that for half of the time intervals in them, the system could sustain the perturbation, whereas for the other half of the interval, perturbation could destabilize the system. To describe these instance **'un(able)'** will be used.

Sensitivity Analysis Table :

	1-12	13-24	25-36	37-48	49-60	61-72	73-84	85-96
CIT	**'able'**	**'unable'**	**'able'**	**'unable'**	**'un(able)'**	**'unable'**	**'un(able)'**	**'unable'**
ICIT	**'able'**	**'unable'**	**'able'**	**'unable'**	**'un(able)'**	**'unable'**	**'un(able)'**	**'unable'**
AKG	**'able'**	**'unable'**	**'able'**	**'unable'**	**'un(able)'**	**'unable'**	**'un(able)'**	**'unable'**
SSA	**'able'**	**'unable'**	**'able'**	**'unable'**	**'un(able)'**	**'unable'**	**'un(able)'**	**'unable'**
SUC	**'able'**	**'unable'**	**'able'**	**'unable'**	**'un(able)'**	**'unable'**	**'un(able)'**	**'unable'**
SCA	**'able'**	**'unable'**	**'able'**	**'unable'**	**'un(able)'**	**'unable'**	**'un(able)'**	**'unable'**
FA	**'able'**	**'unable'**	**'able'**	**'unable'**	**'un(able)'**	**'unable'**	**'un(able)'**	**'unable'**
MAL	**'able'**	**'unable'**	**'able'**	**'unable'**	**'un(able)'**	**'unable'**	**'un(able)'**	**'unable'**
GLY	**'able'**	**'unable'**	**'able'**	**'unable'**	**'un(able)'**	**'unable'**	**'un(able)'**	**'unable'**

Sensitivity Analysis Table (Table-2):

	1-12	13-24	25-36	37-48	49-60	61-72	73-84	85-96
CIT	$[21, 1]$	(100+)	$[3, 1]$	(100+)	$[9, 1]$	(100+)	$[3, 1]$	(100+)
ICIT	$[1, 1]$	(100+)	$[6, 1]$	(100+)	(100+)	(100+)	(100+)	(100+)
AKG	$[1, 1]$	(100+)	$[1, 1]$	(100+)	(100+)	(100+)	(100+)	(100+)
SSA	$[1, 1]$	(100+)	$[9, 1]$	(100+)	(100+)	(100+)	(100+)	(100+)
SUC	$[35, 1]$	(100+)	$[24, 1]$	(100+)	(100+)	(100+)	(100+)	(100+)
SCA	$[3, 1]$	(100+)	$[4, 1]$	(100+)	(100+)	(100+)	(100+)	(100+)
FA	$[3, 1]$	(100+)	$[3, 1]$	(100+)	(100+)	(100+)	(100+)	(100+)
MAL	$[1, 1]$	(100+)	$[3, 1]$	(100+)	(100+)	(100+)	(100+)	(100+)
GLY	$[3, 1]$	(100+)	$[1, 1]$	(100+)	(100+)	(100+)	(100+)	(100+)

Legends (for Sensitivity Analysis Table (Table-2)) :

1) Every cell of the 'Sensitivity Analysis Table (Table-2)' is put in the format $[Perturb_{max}, Perturb_{min}]$, where $Perturb_{max}$ implies the maximum perturbation (perturbation counter), while $Perturb_{min}$ implies the minimum perturbation (perturbation counter) needed to take the TCA cycle to EOL.
2) The (100+) label denotes that even after 100 perturbations, the TCA cycle could not be destabilized to the extent of approaching EOL.

Abbreviations

ICL : isocitrate lyase; AKG : alpha ketoglutarate; CS : citrate synthase; FUM : fumarase; GLY : glyoxylate; ICD : isocitrate dehydrogenase; ICIT : isocitrate; KDH : alpha-ketoglutarate dehydrogenase; KGD : alpha- ketoglutarate decarboxylase; MDH : malate dehydrogenase; MS : malate synthase; ScAS :succinyl-CoA synthetase; SDH : succinate dehydrogenase; SSA : succinic semialdehyde; SUC : succinate; TCA : tricarboxylic acid;

Potential applications of the present study:

Multidimensisonal utiliterian advantages can be drawn from the present work. Here we present with two of these possibilities.

The sensitivity analysis performed here can be of enormous importance to various domains (and sub-domains) in Systems Biology and Synthetic Biology. The values provided in sesitivity analysis table will describe (numerically) the robustness of the system under consideration, with respect to the particular metabolite. It is a known fact that increasing the concentration of a particular metabolite can be used as one of the strategies to kill a biologically functional cell by producing the cytotoxic effect. However an exact measure that quantifies the extent of increament of the concentration of a particular metabolite (to the extent that it causes cytotoxicity) does not exist hitherto. By our approach the exact magnitude of these concentrations can be calculated, which will have global ramification in the sphere of drug design.

Utiliterian aspect of the present work can also be realized in the context of the nascent field of synthetic biology. For example, it still remains a great challenge to achieve the synchronization profile in the artificial cell even after providing it with the necessary building blocks. By our approach the fine tuned concentrations and entropic values, necessarry to ensure the emergence of SP can be calculated. Therefore the present algorithm can be used to produce the benchmarking tools to calibrate the synthetic biology experiments, merely with the information about (time-dependent) concentration profile of the system.

Conclusion :

Negative entropy does not merely imply the presence of order. Instead, it quantifies the probability that new orders with spectrum of scopes and depths, can continuously emerge from the existing paradigm of (fluctuating) order. A crystall at 0 Kelvin, might show perfect order (if we overlook 'zero point energy'), however that same crystall in its perfectly ordered state will fail to create a morsel of richness in pattern that a biological emergence can demonstrate.

The pursuit of our work was to construct a model to probe the origin of this intricately beautiful set of emergent biological patterns; in other words, to construct a method which can unambiguously express the content of negative entropy. A rudimentary model, with primitive constructs, is proposed here; that attempted to measure the (latent) negative entropy present in biological systems. Our model is rudimentary because we could only measure the amount of negative entropy present in TCA cycle (and not in an entire biological cell at its functioning state); it is primitive because the toolset used here are the elementary basics of non-cooperative game theory. But having said that, a consistent and biolgically meaningful set of trends in the set of obtained information proves with sufficient confidence that the same framework can be applied to measure negative entropy in much more involved systems too. Apart from the (obvious) scopes of applicability (described beforehand), attempts like the present one can immediately be linked to causality studies behind understanding why biological systems behave so differently than their physico-chemical analogues.

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